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Characterization of Human Endogenous Retrovirus Type K Virus-like Particles Generated from Recombinant Baculoviruses

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The family of human endogenous retrovirus type K (HERV-K) comprises members with long open reading frames (ORF) for retroviral proteins. The existence of a biologically active provirus with replicative capacities has not yet been demonstrated. To confirm the assumption that HERV-K codes for the previously observed retrovirus-like particles (human teratocarcinoma-derived virus, HTDV) in human teratocarcinoma cells, we have constructed recombinant full-length HERV-K cDNA-based baculoviruses with *gag, pro, pol,* and *env* ORFs. Two viral constructs were used for infections of insect cells, one bearing 67 bp of the 5′ untranslated region upstream of the 5′ splice donor (SD) site and of the retroviral genes, the second omitting the SD sequence. For both recombinant viruses, indirect immunofluorescence and laser scan analyses revealed expression of HERV-K Gag protein. Electron microscopy studies demonstrated efficient production of virus-like particles (VLPs) at the cytoplasmic cell membranes. These VLPs are morphologically identical with the HTDV phenotype. In immunoelectron microscopy of ultrathin frozen sections, anti-HERV-K Gag antibodies specifically reacted with HERV-K VLPs. In Western blots, in addition to the 76-kDa precursor protein, the putative major core protein with an apparent molecular mass of 32 kDa exhibited predominant immunoreactivity with anti-Gag antiserum. In contrast, neither HERV-K Env nor cORF proteins could be detected due to inefficient mRNA splicing. Purified particles from insect cell culture supernatants tested in an ultrasensitive reverse transcriptase assay revealed weak polymerase activity. The data demonstrate that HERV-K codes for retroviral particles of the HTDV phenotype.

INTRODUCTION

The human genome contains a large variety of endogenous retroviral sequences (HERV) of which most are highly defective (reviewed by Wilkinson *et al.,* 1994; Löwer *et al.,* 1996). HERV elements are vertically transmitted in a Mendelian manner and there appears to be no proviral sequence that can give rise to an extracellular life cycle. HERV mRNA is expressed at constitutive levels in numerous cell lines and human tissues. However, expression of HERV proteins appears to be strictly controlled and there is as yet little information on the biological function of these retroviral sequences (Wilkinson *et al.,* 1994; Löwer *et al.,* 1996).

The HERV-K family is present in 30 to 50 copies per haploid human genome (Ono *et al.,* 1986), of which some proviruses display long open reading frames (ORFs) (Ono *et al.,* 1986; reviewed in Löwer *et al.,* 1996; Tönjes *et al.,* 1996). HERV-K10 is a 9.2-kb full-length proviral clone showing disrupted *gag* and *env* regions but containing an ORF of sufficient size to encode a full-length polymerase (*pol*) protein (Ono *et al.,* 1986). Its primer-binding site shows specificity for lysine tRNA as indicated by the suffix K (Larsson *et al.,* 1989). HERV-K proviral sequences are present in the lineage of Old World monkeys and hominoids (Ono *et al.,* 1986; Steinhuber *et al.,* 1995) in which *pol* and *env* gene sequences show extensive conservation (Tönjes and Kurth, 1994).

The phenotype of human teratocarcinoma-derived virus (HTDV) particles produced in teratocarcinoma (TC) cell lines (Kurth *et al.,* 1980; Boller *et al.,* 1983; Löwer *et al.,* 1984) has been linked to complex mRNA expression of HERV-K (Löwer *et al.,* 1993a,b). Antibodies against recombinant HERV-K Gag have been shown to react with HTDV core proteins (Boller *et al.,* 1993; Löwer *et al.,* 1993b). Prokaryotically expressed HERV-K protease (Mueller-Lantzsch *et al.,* 1993; Schommer *et al.,* 1996) and integrase (Kitamura *et al.,* 1996) domains exhibit specific enzyme activities. Reverse transcriptase-like activities could be revealed from TC cell line supernatants using an improved PCR-based reverse transcriptase (RT) assay (Lugert *et al.,* 1996; Tönjes *et al.,* 1996). However, HERV-K *pol* sequences prokaryotically expressed demonstrated no specific RT activities, suggesting that endogenous RT-like enzymes expressed from HERVs probably require more natural conditions in order to be active.

HERV-K type 2 proviruses which harbor a segment of 292 nucleotides at the 5′ end of the *env* gene have the potential to express subgenomic *env* mRNA and doubly spliced transcripts (Löwer *et al.,* 1993a, 1995). HERV-K type 1 genomes devoid of this segment display fused

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pol and *env* genes and merely express unspliced mRNA (Löwer *et al.,* 1995). One of the doubly spliced mRNAs containing most of the 292-bp segment codes for the 14-kDa cORF protein, which accumulates in the nucleolus and shows structural homology to Rev (Löwer *et al.,* 1995). In TC cell lines expression of HERV-K Env proteins is hardly detectable (Tönjes *et al.,* 1997), presumably accounting for the apparent lack of HTDV infectivity (Löwer *et al.,* 1984). In eukaryotic expression systems a full-length HERV-K *env* cDNA from human TC cells displaying an ORF for the surface protein (SU) and a membrane-spanning protein (TM) can be expressed (Tönjes *et al.,* 1997). The recombinant proteins are slightly glycosylated but not cleaved into SU and TM portions neither in insect nor in mammalian cells (Tönjes *et al.,* 1997).

To date, HERV-K is the only known human endogenous retroviral entity with the capacity to code for virus-like particles (VLPs). Although particles that are morphologically indistinguishable from HTDV have been observed in human placentas (Kalter *et al.,* 1973; Vernon *et al.,* 1974; Lyden *et al.,* 1994), no definitive evidence has been provided that they are in fact encoded by HERV-K elements. On the other hand, there is ample evidence that HERV-K sequences code for HTDV particles (reviewed by Löwer *et al.,* 1996; Tönjes *et al.,* 1996); however, no full-length molecular clone of HERV-K has been expressed in recombinant systems so far. In this report the causal relation of HERV-K expression and HTDV phenotype is demonstrated by utilization of the baculovirus expression system (BVES). We show here that baculoviruses recombinant for full-length HERV-K type 2 cDNA elicit expression of HERV-K VLPs in insect cells.

MATERIALS AND METHODS

Isolation of cDNA clones and assembly of full-length HERV expression cassettes

HERV-K cDNAs were isolated from specific libraries of the TC cell line GH using HERV-K *gag* and *pol* probes (Löwer *et al.,* 1993). Plasmid DNAs were digested with appropriate restriction endonucleases (New England Biolabs) and fragments were separated by gel electrophoresis. The HERV-K *gag* containing clone pcG3*gag* (nt 466–3192) and the HERV-K *pol* containing clone pcP23*pol* (nt 3785–6099) were isolated from a random-primed GH cDNA library bidirectionally cloned in Lambda Zap II vector (Stratagene). Clone pcG1*pro* spanning HERV-K protease (*pro*) (nt 1487–4636) was isolated from an oligo(dT)-primed GH cDNA library as was pcK30*env* (nt 6015–9381) which was described previously (Löwer *et al.,* 1995). Numbering of sequences is based on the HERV-K type 2 sequence including three nucleotide insertions as described recently (Löwer *et al.,* 1995). Plasmids pcG3*gag* and pcG1*pro* (cDNA in pBS-SK; Stratagene) were recombined by replacing an ~360-bp *Xba*I (pBS)–*Nar*I (nt 1831 in HERV-K) fragment from pcG1*pro* by an ~1380-bp *Xba*I–*Nar*I fragment from pcG3*gag* generating pcG31 (nt 466–4636) which bears discontinuous *gag* and *pro* ORF (nt 466–4636). An ~3620-bp *Xho*I (nt 1012)–*Xho*I (pBS) fragment was isolated from pcG31 and cloned in pBS-SK yielding pG31X. Sequences pcP23*pol* and pcK30*env* were recombined by insertion of an ~2250-bp *Hin*dIII (pBS)–*Hin*dIII (nt 6031) fragment from pcP23*pol* into the *Hin*dIII site (nt 6031) of pcK30*env*, producing pcPK23/30 (nt 3785–9381). An ~630-bp *Sst*I (pBS)–*Sst*I (nt 4411) fragment was excised from pcPK23/30 and replaced by an ~3400-bp *Sst*I (pBS)–*Sst*I (nt 4411) fragment isolated from pcG31X. Subsequently, an ~7730-bp *Not*I (pBS)–*Sph*I (nt 8737) fragment was excised from the resulting plasmid pcGPK31 and cloned in pGem-5Zf (Promega), generating pcGPK31ΔLTR. All four original cDNA plasmids were fully sequenced by the dideoxy chain termination method except plasmid pcG1*pro* which was partially sequenced from nt 1831 to 4544. Joining restriction sites in expression cassettes were verified by sequencing using appropriate oligonucleotides. Primers were commercially purchased from Eurogentec (Belgium) or produced on an ABI 380B DNA synthesizer in our institute.

The nucleotide sequence data reported in this paper have been submitted to the EMBL nucleotide sequence database and have been assigned the Accession Nos. Y10390 (pcG3*gag*), Y10391 (pcP23*pol*), and Y10392 (pcG1*pro*).

Construction of recombinant baculoviruses

For cloning of recombinant HERV-K baculovirus transfer vectors pBac-HERV-K/mcs, pBac-HERV-K/XB, and pBac-HERV-K/HB, plasmid pcGPK31ΔLTR was linearized using *Bsp*120I (pGem) and the site was blunted employing Klenow enzyme (New England Biolabs). After *Not*I digestion the complete ~7.8-kb insert was isolated and cloned into the *Not*I–*Sma*I sites of baculovirus transfer vector pVL1392 (Pharmingen) yielding pBac-HERV-K/mcs. Vector pBac-HERV-K/XB was generated by partial *Xho*I restriction enzyme digestion of *Bsp*120I-linearized pcGPK31ΔLTR to remove 5′ flanking multiple cloning site (mcs) sequences of pBS-SK and to isolate the ~7.7-kb *Xho*I (nt 1012)–*Bsp*120I (pGem) fragment. Protruding ends were filled in using Klenow enzyme, and after addition of *Not*I linker (New England Biolabs) the fragment was cloned into the *Not*I restriction site of pVL1392 (Pharmingen). Similarly, pBac-HERV-K/HB was produced by partial *Hin*dIII digestion of *Bsp*120I-linearized pcGPK31ΔLTR to remove an additional 67 bp of 5′ untranslated region (UTR) sequences and to isolate the ~7.7-kb *Hin*dIII (nt 1079)–*Bsp*120I (pGem) fragment which, after blunting and *Not*I linker addition, was cloned into the *Not*I restriction site of pVL1392. All expression cassettes bear the

AUG (nt 1112) codon of the HERV-K *gag* gene. In pBac-HERV-K/HB, the splice donor (SD) sequence (nt 1074–1082) was almost entirely destroyed by usage of the *Hin*dIII cloning site.

Insect cell lines SF9 and High Five (Invitrogen) were grown in TNM-FH medium (Sigma) supplemented with 10% fetal calf serum (Seromed). For ease of analysis of secreted proteins after infection, a subcloned cell line of High Five (S-High Five) was grown in SF900 medium (Gibco BRL) without serum. Recombinant HERV-K baculoviruses were generated in SF9 cells as described (Tönjes *et al.,* 1997).

Generation of HERV-K GAG antiserum

Polyclonal antisera against recombinant prokaryotically expressed HERV-K Gag protein were raised in goats as described elsewhere (Boller *et al.,* 1997).

Immunoblot analysis of expressed HERV-K proteins

High Five cells were infected with recombinant HERV-K baculoviruses as described previously (Tönjes *et al.,* 1997) and harvested 24–72 hr postinfection (p.i.) depending on the maximum protein expression of each recombinant virus. Lysates of infected cells and control cells were centrifuged at 14,000 rpm and 4° for 30 min and analyzed by SDS–PAGE (Laemmli, 1970) and immunoblotting (Towbin *et al.,* 1979) using polyvinylidene difluoride membranes (Millipore). Membranes were first incubated with 1:500 to 1:1000 dilutions of goat α-HERV-K Gag (α-Gag), rabbit α-HERV-K SU-Env (Tönjes *et al.,* 1997), and rabbit α-HERV-K cORF (Löwer *et al.,* 1995) antisera or corresponding preimmune sera and subsequently incubated with anti-rabbit or anti-goat IgG-conjugated horseradish peroxidase (1:5000; Amersham) as second antibody. Immunoreactive proteins on membranes were detected using the ECL system (Amersham).

Immunofluorescence

Indirect immunofluorescence for the analysis of HERV-K Gag protein expression was performed essentially as described previously (Boller *et al.,* 1993, 1997). In brief, High Five cells were grown on coverslips and were infected with recombinant baculoviruses in the logarithmic growth phase. Wild-type baculovirus-infected cells and uninfected cells served as controls. After fixation with 2% freshly prepared formaldehyde and permeabilization with 1% Triton X-100 in phosphate-buffered saline (PBS) cells were treated with 1% bovine serum albumin (BSA) to block nonspecific binding sites. HERV-K-specific antisera or corresponding preimmune sera diluted 1:500 to 1:1000 were incubated for 30 min on coverslips and, after washing in PBS, samples were incubated with FITC-labeled anti-rabbit or anti-goat IgG for 20 min. After extensive washing, coverslips were mounted on slides in Moviol (Hoechst, Germany). Preparations were examined utilizing a Zeiss Axiophot microscope equipped with an incident fluorescence illuminator using appropriate filters or a laser scan unit (MRC 600; Bio-Rad) attached to a Zeiss Axiovert fluorescence microscope, employing the CoMOS image analysis software (Bio-Rad).

Electron microscopy (EM)

For plastic embedding, cells were fixed with 2.5% glutaraldehyde in culture medium for 45 min and embedded in Epon resin using standard procedures (Boller *et al.,* 1997). Ultrathin sections were stained with 2% uranyl acetate followed by 2% lead citrate.

For immunoelectron microscopy, cells were prepared as described (Boller *et al.,* 1997). In brief, cells were fixed in a mixture of 2% formaldehyde and 0.1% glutaraldehyde in PBS for 45 min and immersed in 2.3 *M* sucrose, frozen in liquid nitrogen and cut into 100-nm sections on an ultramicrotome (Ultracut 4; Reichert, Austria) using cryoequipment FC4 (Tokuyasu, 1976; Dutton, 1981). Sections were mounted on Formvar-coated nickel grids. After a wash with PBS, grids were treated with 2% BSA and incubated with goat antisera specific for recombinant HERV-K Gag or corresponding preimmune sera, both diluted 1:1000. After a rinse in PBS, grids were incubated with rabbit anti-goat IgG, coupled to 10-nm gold particles (BioCell, UK). Finally, sections were embedded in methylcellulose and stained according to Griffiths *et al.* (1983).

All EM preparations were examined in a Zeiss EM 902 electron microscope using the ESI mode.

RT PCR analyses

For isolation of RNA from particulate material, subsequent to removal of cells and cellular debris by centrifugation at 4000 *g* for 10 min, 5 ml of tissue culture supernatants of infected High Five cells 3 days p.i. or uninfected controls was filtered through Minisart NML filter units (0.2 μm; Sartorius) to remove cell contaminants. HTDV-expressing human TC cells GH and human fibroblastoid cells MRC5 served as positive, i.e., HERV-K-expressing, and negative controls, respectively. Samples were further subjected to ultracentrifugation at 100,000 *g* for 1 hr at 4° in a SW55 rotor (Beckman). Pellets were dissolved in PBS, and RNA was extracted using Trizol reagent (Life Technologies) and subsequently digested with RNase-free DNase I (Boehringer) at 0.1 u per 1 μl of reaction volume. After heat inactivation of DNase I at 65° for 10 min, phenol extractions, and ethanol precipitation, RNA was dissolved in 10 μl DEPC-treated water. RT PCR was performed essentially as described (Simpson *et al.,* 1996) using oligonucleotide ABDPOR (Medstrand *et al.,* 1992) as primer for cDNA synthesis and ABDPOL (Medstrand *et al.,* 1992) and ABDPOR primers for PCR amplifications.

First-strand reactions omitting RT served as internal controls to monitor DNA contaminations. The ~296-bp amplicons were separated on 1.5% agarose gels.

Cellular RNA was isolated according to standard techniques (Sambrook *et al.,* 1989). Full-length HERV-K mRNA in insect cells was detected by PCR amplification of random-primed cDNA using oligonucleotides Bac-F (TTTACTGTTTTCGTAACAGTTTTG) for the transcribed polyhedrin promoter sequence (nt 4049–4072 in pVL1392) and S21-R (GCTTGTTTTAGTTCCTTACC) for HERV-K *gag* (nt 1320–1301). Spliced HERV-K mRNA was amplified using primers Bac-F and Env-R (CTACTTCTTTCTACACAGACACAG; nt 8560–8537). TC cell line GH mRNA served as a positive control using HERV-K-specific primer PBS-F (CTGGTGCCCAACGTGGAGGCTTTTCTCTAG; nt 970–999) instead of Bac-F.

RT assays

Reverse transcriptase activity was detected by using an ultrasensitive PCR-based assay including activated DNA (5 μg) to suppress false-positive RT-like activities as described (Lugert *et al.,* 1996). Samples were harvested from tissue culture supernatants (see before) and particulate material was dissolved in 20 μl buffer A (50 m*M* KCl, 25 m*M* Tris–HCl, pH 7.5, 5 m*M* DTT, 0.25 m*M* EDTA, 0.025% Triton X-100, 50% glycerol) of which 1–2 μl were used for each analysis.

RESULTS

Characterization of HERV-K sequences

Four overlapping HERV-K cDNA clones isolated from two TC libraries were used for generation of recombinant baculovirus vectors. These sequences encompassing *gag, pro, pol,* and *env* ORFs were selected for the composite full-length HERV-K cDNA after individual protein expression in appropriate systems. The *gag* ORF (pcG3*gag*) was expressed as an ~80-kDa C-terminal His-tag fusion protein using vector pET15b (Novagen) in *Escherichia coli* (not shown). The HERV-K protease sequence of prokaryotically expressed clone pcG1*pro* (in pET15b) demonstrated autocatalytic activity and cleavage of coexpressed HERV-K Gag precursor into major core proteins (not shown). His-tagged (pET15b) or anthranilate synthetase fusion proteins of the *pol* ORF derived from pcP23*pol* were expressed in *E.coli* (not shown). Purified fusion proteins did not reveal RT activities when analyzed in a modified product enhanced reverse transcriptase assay (Lugert *et al.,* 1996; data not shown). The Env protein encoded by clone pcK30*env* which was described previously (Löwer *et al.,* 1995) has been separately expressed in insect and mammalian cells (Tönjes *et al.,* 1997). It could be shown that in both systems HERV-K Env with an apparent molecular mass of 80–90 kDa is glycosylated but is not cleaved into SU and TM portions and is not translocated to the outer cell membrane, although the signal peptide (SP) is cleaved (Tönjes *et al.,* 1997). These four cDNA clones were recombined and three full-length retrovirus sequence cassettes differing in extent of their 5′ UTR were cloned into baculovirus transfer vector pVL1392 (Fig. 1).

Expression of HERV-K VLPs in insect cells

The BVES enables expression and processing of foreign proteins in insect cells (O'Reilly *et al.,* 1992). We have generated three recombinant baculoviruses in order to study eukaryotic expression of HERV-K proteins. Viral vectors Bac-HERV-K/mcs, Bac-HERV-K/XB, and Bac-HERV-K/HB bearing HERV-K *gag, pro, pol,* and *env* genes and devoid of LTR sequences (Fig. 1) were used for infections of High Five insect cells. Bac-HERV-K/mcs harboring a stretch of restriction sites (mcs) derived from the prokaryotic cloning vector upstream of the HERV-K cassette (nt 1012–8737) did not reveal detectable HERV-K protein expression by means of immunoblotting and indirect immunofluorescence using different antisera (not shown). Most likely, this effect was due to the length of the leader region and its potential to form stable stem–loop structures. By contrast, viral vector Bac-HERV-K/XB lacking the mcs sequence and merely containing 100 bp of 5′ UTR upstream of the *gag* initiation codon (nt 1112) showed strong expression of HERV-K GAG proteins at the cytoplasmic membranes (Fig. 2a). Similarly, Bac-HERV-K/HB which retained 33 bp of 5′ UTR and lacks the SD sequence demonstrated strongest Gag expression in insect cells 48–72 hr p.i. (Fig. 2b). These reactions were specific as neither uninfected (Fig. 2c) nor wild-type baculovirus (not shown)-infected cells incubated with α-Gag antibodies nor infected cells incubated with preimmune serum (not shown) showed distinct staining. No base levels of HERV-K Env or cORF proteins could be found in infected cells, neither in Western blots nor by indirect immunofluorescence. RT PCR analyses of cellular RNA revealed the presence of full-length HERV-K mRNA but no spliced *env*- or *cORF*-specific transcripts were found in the case of Bac-HERV-K/HB lacking the SD sequence. In Bac-HERV-K/XB infected cells, low *cORF* mRNA levels were detectable (not shown).

Immunoblotting of insect cell lysates and tissue culture supernatants confirmed the proper expression of HERV-K Gag proteins (Fig. 3). Virus Bac-HERV-K/XB evoked expression of 76-kDa Gag precursor protein and processed proteins including the 32-kDa major Gag 24 hr p.i. (Fig. 3, lane 2) which became even more prominent 48–72 hr p.i. (Fig. 3, lanes 3 and 4). In tissue culture supernatants no Gag precursor was found but the appearance of processed proteins paralleled the expression observed in cell lysates (Fig. 3, lanes 6–8). The same results were obtained with recombinant virus Bac-HERV-K/HB (not shown). As a reference for HERV-K Gag

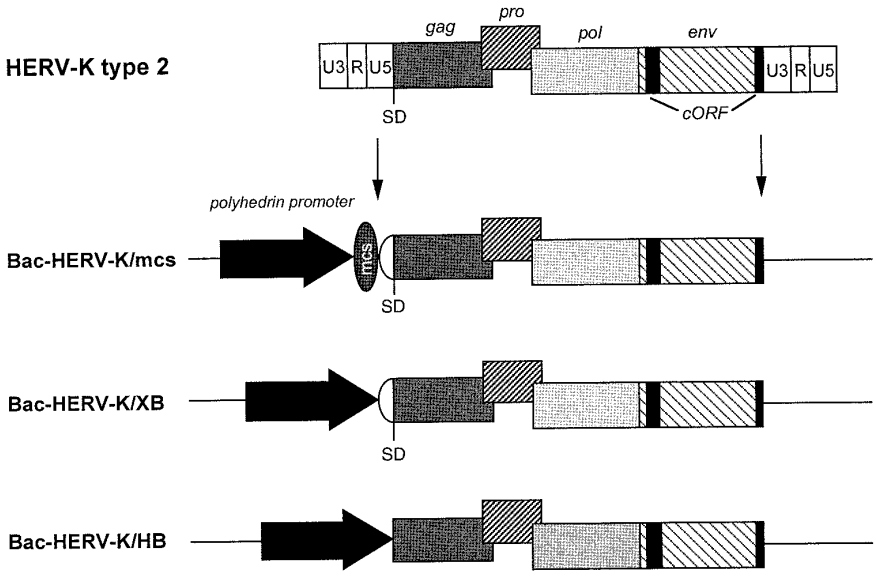

FIG. 1. Structures of recombinant HERV-K baculoviruses. Bac-HERV-K/mcs, full-length HERV-K including plasmid vector mcs sequence and 100 bp of 5′ UTR upstream of *gag, pro, pol,* and *env* genes; Bac-HERV-K/XB, full-length HERV-K including 100 bp of 5′ UTR; and Bac-HERV-K/HB, full-length HERV-K including 33 bp of 5′ UTR but devoid of SD site. Section of HERV-K type 2 prototype provirus (Löwer *et al.,* 1995) is given for comparison.

precursor protein a prokaryotically expressed *gag* cDNA derived from clone pcGU1*gag* (Boller *et al.,* 1997) was used which revealed an apparent molecular weight of 80-kDa (Fig. 3, lane 9). A similar staining pattern was found in sedimented viral material isolated from tissue culture supernatants by ultracentrifugation. Both viruses Bac-HERV-K/XB (Fig. 3, lane 11) and Bac-HERV-K/HB (Fig. 3, lane 12) elicited production of major Gag and specific intermediate proteins when compared with wild-type baculovirus (Fig. 3, lane 10).

Electron microscopic observation of HERV-K VLPs

Electron microscopy studies of ultrathin sections from plastic-embedded insect cells supported the hypothesis that maturation of HERV-K Gag proceeded in retroviral particles (Fig. 4). Both recombinant viruses Bac-HERV-K/XB and Bac-HERV-K/HB evoked efficient production of VLPs at the cytoplasmic membranes (Figs. 4a and 4b). These VLPs were structurally identical to HTDV particles produced by TC cell lines: Their diameter was ~110 nm, they exhibited an electron-dense area between core and membrane, and no spikes or knobs were detectable on the surface. Free mature particles with collapsed cores were virtually not observed. In contrast, a recombinant virus bearing only the HERV-K *gag* gene (Ac-GAG; Sauter *et al.,* 1995) generated plaque-like structures underneath the cell membrane (Fig. 4c). No VLP phenotypes were observed for Ac-GAG, indicating that expression of

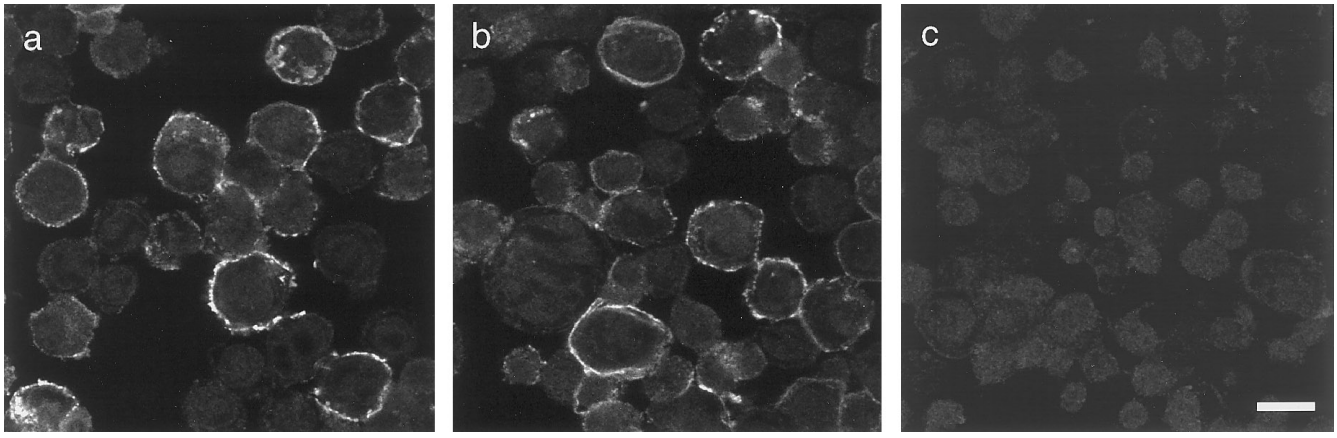

FIG. 2. Immunofluorescence labeling and laser scan microscopy analysis of insect cells infected with recombinant HERV-K baculoviruses. High Five cells were fixed 48 hr p.i. and incubated with α-HERV-K Gag antisera. (a) Cells infected with Bac-HERV-K/XB, (b) cells infected with Bac-HERV-K/HB, and (c) uninfected cells incubated with α-Gag (note that these cells exhibit a much smaller diameter). Scale bar represents 25 μm.

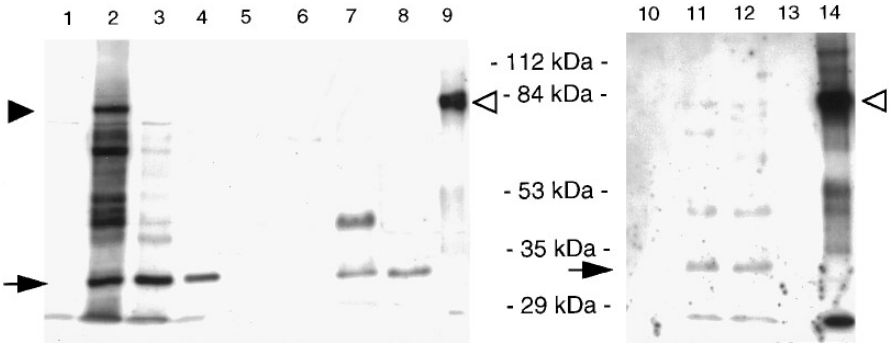

FIG. 3. Expression of HERV-K Gag protein in insect cells. Immunoblots of insect cells infected with recombinant HERV-K baculoviruses. Lanes 1–4, cell lysates of uninfected (lane 1) and Bac-cGPK31/XB-infected High Five cells (lanes 2–4; 24, 48, and 72 hr p.i.); lanes 5–8, tissue culture supernatants of High Five cells infected with wild-type baculovirus (lane 5) and with Bac-cGPK31/XB (lanes 6–8; 24, 48, and 72 hr p.i.); lanes 10–13, virus pellets from tissue culture supernatants (48 hr p.i.); lane 10, High Five cells infected with wild-type baculovirus; lane 11, High Five cells infected with Bac-cGPK31/XB; lane 12, High Five cells infected with Bac-cGPK31/HB; lane 13, uninfected High Five cells; lanes 9 and 14, prokaryotically expressed full-length HERV-K Gag protein (cGU1*gag;* Boller *et al.,* 1997). Samples were separated by 10% SDS–PAGE. Blots were incubated with α-HERV-K Gag antisera. Open triangle indicates prokaryotically expressed Gag, black triangle indicates Gag precursor, and arrows denote major Gag proteins.

HERV-K Gag–Protease precursor is required for VLP formation *in vivo.* The specificities of Gag antibody reactions were further substantiated by immunoelectron microscopy of ultrathin frozen sections in which only HERV-K VLPs produced from recombinant viruses Bac-HERV-K/XB and Bac-HERV-K/HB were labeled (Figs. 5a–5e). The immunogold staining of HERV-K Gag proteins expressed from virus Ac-GAG (Fig. 5f) confirmed the phenotype observed in classical ultrathin sections.

Analyses of the budding process of HERV-K VLPs generated in insect cells (Figs. 6a–6d) revealed identical patterns when compared with budding sequences of HTDV particles observed in human TC cells (Figs. 6e–6h), showing the typical C-type budding process during which virus cores are assembled at the cytoplasmic membrane.

The possible colocalization of VLPs and HERV-K Env proteins was investigated by coinfections of recombinant full-length viruses and Env-expressing virus Bac-Ex30 (Tönjes *et al.,* 1997). By means of immunoelectron microscopy analysis no Env was found associated with HERV-K VLPs (not shown).

HERV-K VLPs contain RNA and RT

The presence of HERV-K RNA in VLPs was examined by RT PCR analysis of RNA isolated from particulate material of insect tissue culture supernatants. As recombinant HERV-K baculoviruses were copurified with HERV-K VLPs by sterile filtration and ultracentrifugation, the isolated nucleic acids were treated with RNase-free DNase and subjected to RT PCR using HERV-K *pol*-specific primers. VLPs generated by Bac-HERV-K/XB and Bac-HERV-K/HB revealed amplicons of expected size (296 bp; Fig. 7, lanes 1 and 2). RNA isolated from particulate fractions of the HTDV-producing and HERV-K mRNA-expressing TC cell line GH revealed the same result (Fig. 7, lane 5). No amplicons were obtained from human fibroblast cell line MRC (Fig. 7, lane 6), which does not express particles and/or HERV-K, and from insect cell controls (Fig. 7, lanes 3 and 4). Appropriate internal controls omitting RT for cDNA synthesis did not yield amplificates (not shown).

Reverse transcriptase activities associated with VLPs were investigated employing a PCR-based protocol as established by Silver *et al.* (1993) and further improved by including activated DNA to suppress false-positive RT-like activities (Lugert *et al.,* 1996). These assays demonstrated weak but specific RT activities for purified VLPs produced by the recombinant viruses Bac-HERV-K/XB and Bac-HERV-K/HB (Fig. 8, lanes 1 and 2), but not for wild-type baculovirus (Fig. 8, lane 3). HTDV particles purified from TC cell supernatants (Fig. 8, lane 4) but not from supernatants from MRC fibroblast cells (Fig. 8, lane 5) demonstrated RT activity in the range of the internal MuLV RT control (Fig. 8, lane 6).

DISCUSSION

The BVES was developed as a tool that allows the introduction, maintenance, and high-level expression of foreign genes (Summer and Smith, 1987; O'Reilly *et al.,* 1992). Genes from numerous human and animal viruses have been inserted into baculovirus vectors and expressed successfully in the insect cell expression system. Several studies have shown that, for instance, structural proteins from human immunodeficiency virus (Wagner *et al.,* 1996), bluetongue virus (French and Roy, 1990; LeBlois and Roy, 1993), rotavirus (Crawford *et al.,* 1994; Zeng *et al.,* 1996), and herpes simplex virus (Trus *et al.,* 1995) expressed from baculovirus vectors, singly or in coinfections, assemble into VLPs. As most of the

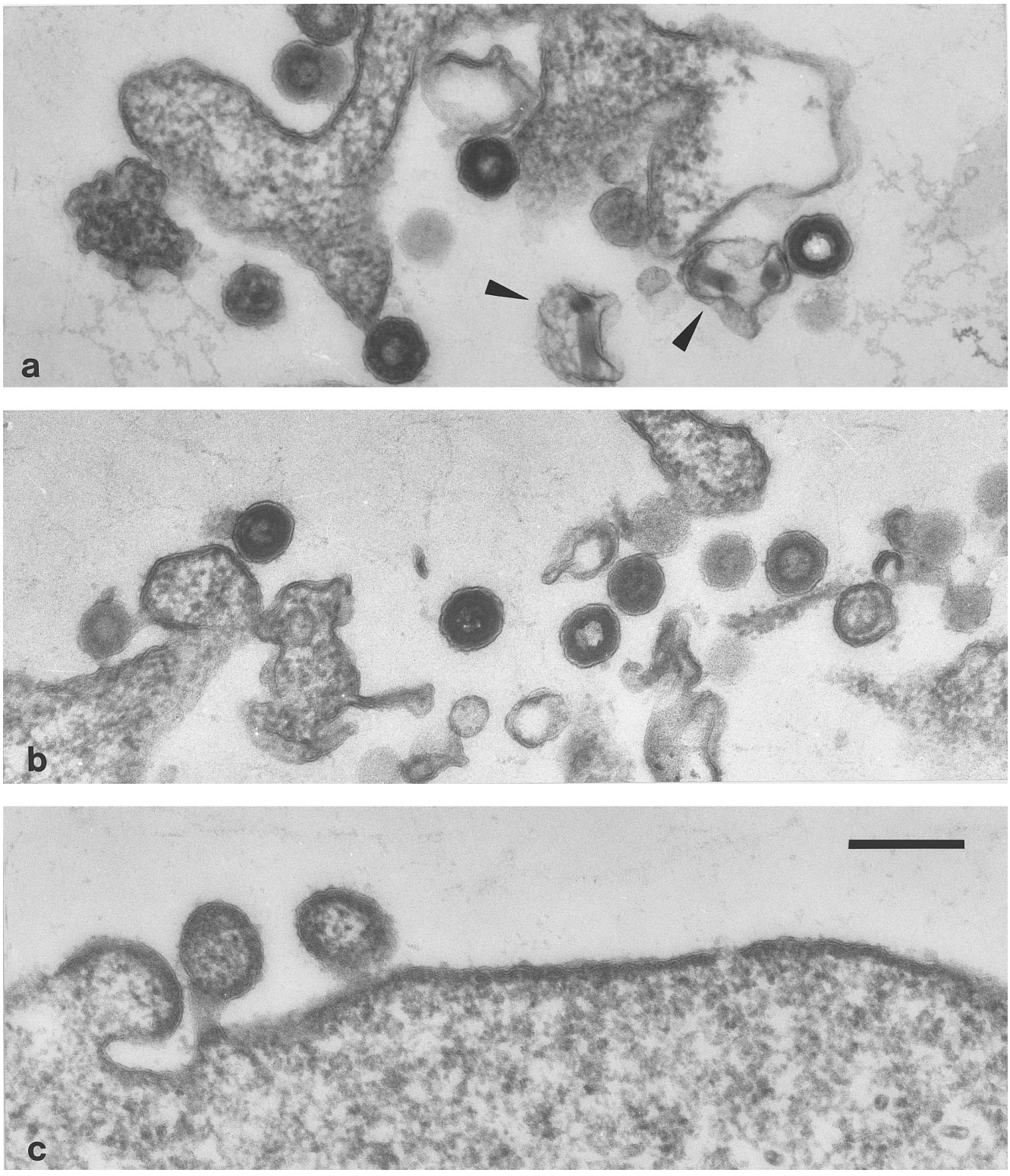

FIG. 4. Morphology of recombinant HERV-K viruses in ultrathin sections of infected High Five cells. (a) Cells infected with virus Bac-HERV-K/XB, (b) Bac-HERV-K/HB, and (c) AcGAG. The diameter of both HERV-K retroviral particles (XB and HB) is approx 110 nm. Viruses XB and HB are morphologically identical. Arrows (a) denote baculovirus particles; bar (c) represents 250 nm.

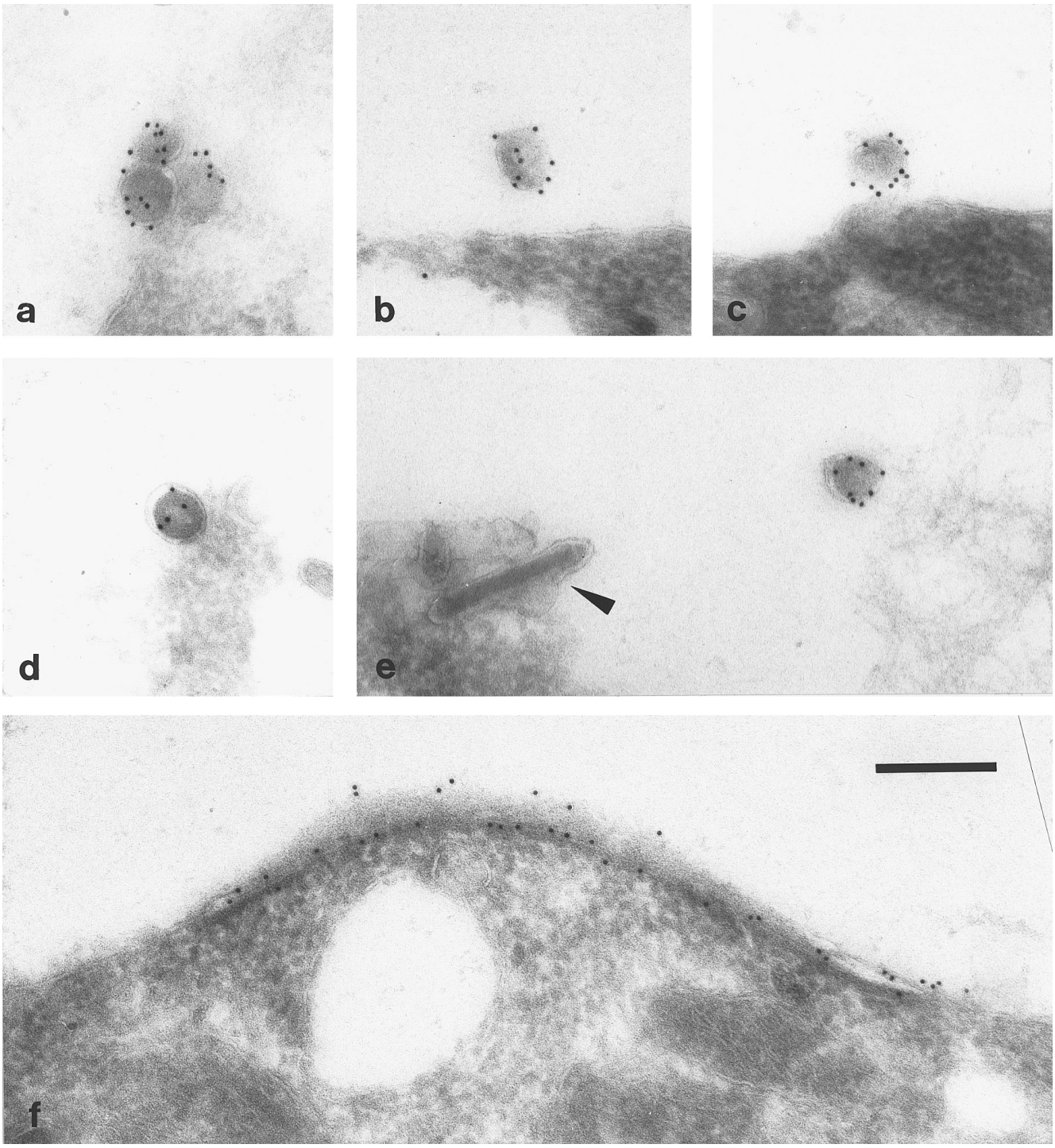

FIG. 5. Immunoelectron microscopic analysis of HERV-K Gag protein in insect cells infected with recombinant HERV-K baculoviruses. (a–c) Cells infected with virus Bac-HERV-K/XB. (d and e) Cells infected with virus Bac-HERV-K/HB. (f) Cells infected with virus Ac-GAG. Ultrathin frozen sections of High Five cells were incubated with α-HERV-K Gag antisera and bound antibodies were subsequently detected with anti-goat IgG (conjugated to 10-nm gold particles). Arrow (e) denotes baculovirus particle; bar (f) represents 250 nm.

expressed proteins maintain the biochemical properties and immunogenicity of their authentic viral counterpart proteins, vaccination protocols for animals based on purified VLPs have been established demonstrating new pathways of immunogen production and protection against viruses (Roy *et al.,* 1994; Wagner *et al.,* 1995, 1996).

We have adopted the powerful BVES to demonstrate

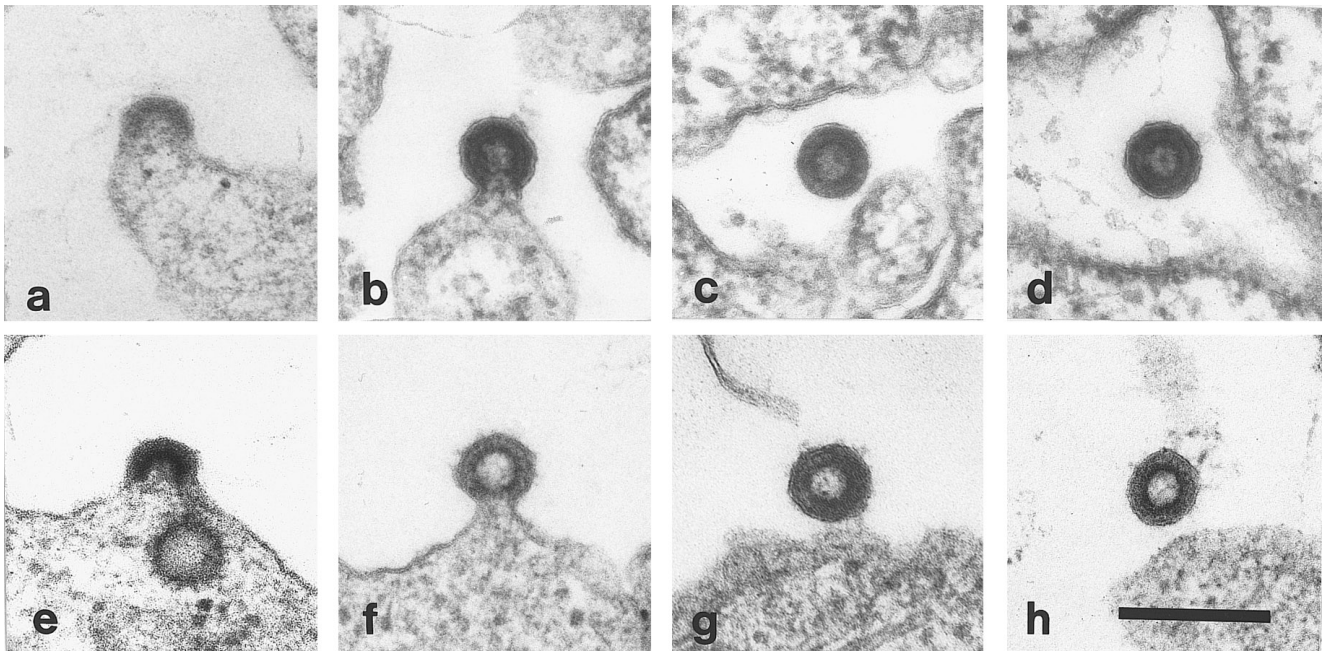

FIG. 6. Budding of recombinant HERV-K virus-like particles in insect cells (a–d) compared with HTDV in human teratocarcinoma cells (e–h). (a, c, and d) Cells infected with virus Bac-HERV-K/XB and (b) Bac-HERV-K/HB. There are no morphological differences between recombinant HERV-K particles and HTDV. Free mature particles with condensed cores as normally seen with infectious retroviruses are virtually not observed.

unequivocally that expression of a full-length molecular clone of HERV-K yields VLPs that are identical with the HTDV phenotype observed in human TC cells. The initial correlation between production of these retrovirus-like particles and expression of HERV-K sequences was established by the advent of the specifically designed RU5-PCR technique (Löwer *et al.,* 1993b). It was shown that, in addition to HERV-H sequences, HERV-K endogenous retroviral elements are highly expressed in TC cell lines and that they most likely code for HTDV particles (Boller *et al.,* 1993; Löwer *et al.,* 1993a). The mRNA expression pattern of HERV-K resembles that of complexly regulated retroviruses, in which full-length transcripts and subgenomic *env* and multiply spliced small RNAs occur (Löwer *et al.,* 1993a). In the course of these analyses a new gene represented by a 1.8-kb doubly spliced mRNA designated *cORF* was identified (Löwer *et al.,* 1993a). This transcript encompasses most of the type-2-specific Env SP and in parallel encodes the cORF protein (Löwer *et al.,* 1995).

We show here that recombinant full-length HERV-K baculoviruses produce phenocopies of the TC cell line-restricted HTDV phenotype when expressed in insect cells. The HERV-K VLPs as HTDV particles lack an electron-lucent space between viral core and envelope, and mature forms with collapsed cores are extremely rare. In addition to these findings the insufficient production

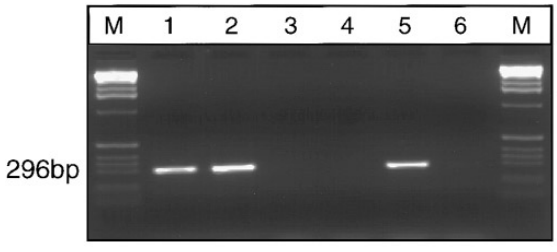

FIG. 7. RT PCR on RNA isolated from particulate material of tissue culture supernatants of insect cells and human teratocarcinoma and fibroblastoid cells. Lane 1, Bac-HERV-K/XB-infected insect cells; lane 2, Bac-HERV-K/HB-infected insect cells; lane 3, baculovirus wild-type-infected cells; lane 4, uninfected insect cells; lane 5, human teratocarcinoma cell line GH; lane 6, human fibroblastoid cell line MRC5; M, molecular weight marker. The 296-bp amplicon is indicated. No signal was detectable in the minus RT lanes (not shown).

FIG. 8. Reverse transcriptase assay using particulate material of tissue culture supernatants from insect cells and human teratocarcinoma and fibroblastoid cells. Lane 1, Bac-HERV-K/XB-infected insect cells; lane 2, Bac-HERV-K/HB-infected insect cells; lane 3, baculovirus wild-type-infected cells; lane 4, human teratocarcinoma cell line GH; lane 5, human fibroblastoid cell line MRC5; lane 6, MuLV RT (0.1 mU); M, molecular weight marker.

of Env in TC cell lines (Tönjes *et al.,* 1997) may be another cause for the apparent lack of infectivity. The production of Gag in VLP-expressing insect cells appears to be identical to the morphological and biochemical patterns observed in TC cells. Particularly, the presence of processed Gag proteins in insect cells infected with both types of recombinant full-length HERV-K baculoviruses, with or without the SD sequence motif upstream of the Gag AUG, suggests the activity of a functional HERV-K protease translated from full-length mRNA by ribosomal frameshifting. This finding is corroborated by the fact that singly expressed HERV-K Gag in insect cells merely produces a cytoplasmic membrane-bound protein layer but no VLP structures.

Despite of the presence of intact HERV-K *env* and *cORF* genes in recombinant viruses, expression of Env and cORF proteins was not detectable in this experimental setup. This phenomenon is intrinsic to the BVES where only low levels of mRNA splicing have been reported for heterologous gene expression (Jeang *et al.,* 1987; Iatrou *et al.,* 1989). Furthermore, strong expression of proteins from spliced mRNAs has not been observed. On the other hand, we have shown previously that both HERV-K genes, *env* and the colinear *cORF,* can be efficiently expressed as single gene cassettes in the BVES leading to production of Env and cORF proteins (Tönjes *et al.,* 1997). Hence, the HTDV situation in TC cell lines and the production of recombinant HERV-K VLPs in insect cells are almost identical regarding Env expression. The existence of the SD sequence in recombinant virus Bac-HERV-K/XB did not quantitatively cause a different pattern of expression, indicating that splicing which is normally mediated by this motif in TC cells (Löwer *et al.,* 1993b) was not enhanced in insect cells. Independent expression of HERV-K Env from coinfected virus Bac-Ex30 (Tönjes *et al.,* 1997) did not yield colocalization with VLPs at insect cell membranes. Most likely, this result is due to the fact that Env is not cleaved to SU and TM portions and that it is not detectable on the surface of insect cells (Tönjes *et al.,* 1997).

An indirect correlation between expression of HERV-K *pol* sequences and RT activity has been provided for particulate material isolated from TC cell line supernatants and for Pol-immunoreactive proteins isolated from TC cell membranes, indicating a Mg^{2+}-dependent enzyme (Tönjes *et al.,* 1996). However, purified recombinant HERV-K Pol proteins showed no enzymatic activities. As shown here, HERV-K VLPs isolated from insect tissue culture supernatants revealed specific but much lower RT activities when compared with HTDV. It is not clear whether this result is due, e.g., to the genetic construct and to the structure of recombinant VLPs or to the *pol* cDNA involved. This sequence was found to be one of two almost identical clones originally isolated from the TC cell line GH. No other expressed endogenous retroviral *pol* sequences with ORFs have been found in TC cells. Furthermore, quantitative aspects like production rates of particles in these two systems might play a role.

Boyd and coworkers have recently shown that VLPs isolated from human placenta bear RT activity and contain HERV-K-related sequences with ORFs for *pol* (Simpson *et al.,* 1996). As this tissue is known to produce VLPs (Kalter *et al.,* 1973) which morphologically resemble HTDV, it is very likely that HERV-K is the only candidate for supplying RT of endogenous retroviral origin. The defective human endogenous retrovirus HERV-R (reviewed in Wilkinson *et al.,* 1994) which highly expresses Env protein in placenta (Venables *et al.,* 1995) can be excluded because of disrupted *pol* sequences. However, direct evidence that VLPs in placenta are in fact encoded by HERV-K proviruses has not been provided. So far, *in situ* analysis showed anti-HERV-K Gag-immunoreacting proteins only in the cytoplasm of testicular tumors (Sauter *et al.,* 1995), but particles have not yet been described for this tissue.

We have shown in this communication that expression of full-length HERV-K cDNA in insect cells leads to formation of VLPs of the HTDV phenotype. Similar experiments based on appropriate mammalian expression vectors are planned in order to study the particle production and coexpression of polymerase and envelope proteins more closely. The involvement of homologous HERV-K LTR elements will be necessary to investigate the requirements for the propagation of HERV-K particles in suitable heterologous tissue culture systems.

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